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Hybrid PLLA/PCL nanofibrous scaffold can improve biochemical and molecular markers of Human Induced Pluripotent Stem Cells (hiPSCs) derived Hepatocyte-like cells

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Running Title

Differentiation of hiPSCs to hepatocyte-like cells on PLLA/PCL

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ABSTRACT

A suitable alternative strategy for liver transplantation is the use of nanofibrous scaffolds together with stem cells. In this study, a random hybrid of poly-L-lactic acid (PLLA) and polycaprolactone (PCL) was used as a three-dimensional (3D) culture for hepatocyte-like cells (HLCs) differentiation and compared with routine culture (2D). The expression of the endodermal marker, FOXA2, was assessed on day 3 and the hepatic markers; albumin (ALB), alpha-1 antitrypsin (AAT), and cytokeratin-18 (CK-18) were evaluated on day 18 by (qPCR). As well as, albumin, alpha-fetoprotein, and low-density lipoprotein (LDL) uptake were evaluated by immunocytochemistry; moreover Periodic Acid-Schiff and Oil red were done by cell staining. In addition, alpha-fetoprotein (AFP) and urea production were evaluated by chemiluminescence and colorimetric assays. Light and Scanning Electron Microscopy (SEM) showed changes in the cells in 2D and 3D models. The gene expression of hepatic markers was significantly higher in the three-dimensional cultures. In addition, immunocytochemistry and cell staining showed that albumin, alpha-fetoprotein, LDL-uptake, Periodic Acid-Schiff, and Oil red were expressed in both cells derived on 2D and 3D. Furthermore, the evaluation of alpha-fetoprotein and urea secretion was significantly different between 2D and 3D strategies. These findings suggest that functionally cells cultured on a PLLA/PCL scaffold may be suitable for cell therapy and regenerative medicine.

Keywords: Human induced pluripotent stem cells, Hepatocyte, PLLA/PCL, Scaffold, and Nanofiber

Introduction

Liver transplantation is currently the best treatment for patients at the final stage of liver failure (Yu et al., 2012). However, the number of donor organs is very limited. Hence, the use of novel approaches such as stem cells, especially human induced pluripotent stem cells (hiPSCs) and artificial cell engineering for regenerative medicine have been proposed as a new alternative cell therapy to overcome the shortage of transplantable livers. hiPS cells are human-specific cells with unlimited pluripotent like-embryonic stem cell properties that they are ideal cells for differentiation (Mahboudi et al., 2017), and they may provide a source of hepatocyte-like cells (HLCs) that may provide an alternative to transplantation therapy in regenerative medicine. Moreover, the properties of nanofibrous scaffolds used in tissue engineering are similar to the natural extracellular matrix (ECM) and this subject is vital for cell-matrix, cell-cell and cell-scaffold contacts for the regulation of cell growth and differentiation process which can be adopted by the body system. Nanofibrous scaffolds are synthetic biocompatible scaffolds which have topographic structures that can be fabricated to mimic the structures of natural ECM (Mashhadikhan et al., 2015). Preliminary studies have used various scaffolds such as polyethersulfone (PES) (Hashemi et al., 2009; Kazemnejad et al., 2009; Mahmoodinia Maymand et al., 2017), PLLA (Feng et al., 2010; Ghaedi et al., 2012) and PCL/collagen/PES (Mahmoodinia Maymand et al., 2017) for hepatocyte differentiation. However, these scaffolds have limitations; for example Zhang et al., 2005 reported that PCL nanofibers need effective hybridization with bioactive molecules (Zhang et al., 2005). As well as, PES is a non-biodegradable and suitable polymer requested to repair hard tissues like bone and cartilage. Likewise, researchers are less likely to use pure PLLA because of poor biocompatibility. Most importantly, scaffolds fabricated of single polymers display poor mechanical properties and are not easy to handle (Kazemnejad et al., 2007). Until recently, studies have been conducted on non-embryonic-like stem cells. To overcome

these limitations, many reports have indicated the possible applications of PLLA/PCL blends (Chen et al., 2013; Mobarra et al., 2018), and generally, it has been specified that PLLA/PCL hybrid fibers are nano-bio degradable, cost-effective, flexible and biocompatible polymers that their structure is similar to ECM and important that this polymer is ideal for the differentiation of soft tissues (Shakhssalim et al., 2017). Various techniques have emerged for the fabrication of nanofibers, But generally, electrospin is most commonly used for the fabrication of nanofibers because of low cost, the alter of solution composition and simplicity in biological manipulation (Zhang et al., 2007). Also, the use of this process has the potential to produce scaffold polymers on a large scale. Following this pattern and the production of induced cell-based tissue regeneration, in the present study, to investigate the capacity of PCL/PLLA nanofibers to support the successful differentiation of the hiPSCs to HLCs, we evaluated the expression of biochemical and molecular liver markers. Under in vitro conditions, results showed that 3D hybrid scaffold compared to 2D culture can provide the better function of new hepatocytes for hiPS stem cell therapy and regenerative medicine.

Method and material

The culture of human induced pluripotent stem cells and embryonic body formation

Culture and expansion of hiPSCs were undertaken as previously described (Mobarra et al., 2014). These embryonic-like stem cells were seeded on to mouse embryonic fibroblast (MEF) feeder layer inactivated with mitomycin-C (Sigma, St. Louis, MO) in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM F-12) enriched with knockout serum replacement, 100 μ M nonessential amino acids, 2 mM L-glutamine, 100 μ M 2-mercaptoethanol and 4 ng/ml basic fibroblast growth factor (bFGF) (all from Invitrogen). The cells were incubated at 37°C under 95% humidity and 5% CO₂ for embryonic body (EB) formation. Aggregated hiPSCs colonies were detached with collagenase IV (Gibco, 15140-

122) and transferred into four-well non-attachment plates containing complete medium without bFGF for differentiation.

The differentiation of hiPSCs to HLCs

EB cells were subcultured in two groups: group A was expanded on the nanofibrous hybrid membrane and the next group was transferred to gelatin-coated tissue culture plate. Briefly, to induce endoderm, for one day EBs plated in serum-free RPMI 1640 medium (Gibco, 11875119) supplemented with B27 (Gibco, 0080085SA) and 100 ng/ml activin A (Stemgent 03-001). Insulin-transferrin-selenium (ITS, Gibco, 41400045) was added to this medium for the following two days. The medium was replaced with Hepatocyte Culture Medium (HCM, Lonza, CC-3198) enriched with 2% fetal bovine serum (FBS), 20 ng/ml hepatocyte growth factor (HGF, R&D, 294-HGN-005) and 20 ng/ml fibroblast growth factor-4 (FGF-4, R&D, 235-F4-025) to produce pre-mature hepatocytes. The medium was changed every 2 days. After 8 days of culture, to mature the hepatic cells, cultured cells were treated with 5% FBS, 20 ng/ml oncostatin M (OSM, R&D, 295-OM-010) plus 0.1 μ M dexamethasone (Dex, R&D, 2749/10), 1% non-essential amino acids and L-glutamine that added to HCM on gelatin-coated plates and PLLA/PCL nanofibrous scaffold membrane for additional 9 days. In parallel, we added hiPSCs media for the control group.

PLLA/PCL fabrication and oxygen treatment

Random nanofibrous PLLA/PCL scaffold was fabricated via electrospinning in the Stem Cell Technology Research Center. Briefly, PLLA 4% and PCL 8% powder dissolved in chloroform, then N, N-dimethylformamide (DMF) as the second solvent was added and the mixture was homogenized in the needle. After that, the collector was placed at a distance of 15 cm from the needle. A polymer solution with a high voltage potential (20 kV) was forced through the needle and collected as nanofibers on the rotating cylinder. To increase the

hydrophilic feature, the random membrane was treated with pure oxygen in a plasma generator under low 44 KHz frequency in cylindrical quartz reactor (Diener Electronics, Germany) at 0.4 mbar pressure and then the glow discharge was performed for 3 min. Nanofibrous sheets were punched into discs with a diameter conforming to the 4-well plates, afterward were sterilized for 30 min and put overnight in the complete culture medium.

RNA extraction and Real-Time PCR processing

Total RNA was extracted on days 3 and 18 in 2D and three-dimensional cultures, as well as a control group using TRIzol Reagent (Gibco, 15596018). For the reverse transcriptase reaction, approximately 3 µg of total RNA was used with the revert aid first strand cDNA synthesis Kit (Fermentas, k1621) and oligo dT primers according to the manufacturer's instructions. The cDNA was amplified by using Takara SYBR Premix ExTaq Master Mix (RR820A). Gene expression levels were quantified by the ABI Light Cycler (ABI step one) and analyzed by rest 2009. Target gene expression values were normalized to the signal get from housekeeping gene beta-Actin mRNA (ActB) and calibrated to the hiPSCs. Primers and product lengths are presented in Table 1.

Immunocytochemistry staining

Cells were rinsed twice with PBS and fixed with 4% paraformaldehyde (Sigma, 30525-89-4) at room temperature (RT) for 20 min. Afterward, the cells were washed and permeabilized with 3% Triton X-100 in phosphate-buffered saline (PBS). Then, the fixed cells were incubated with primary-mouse antibodies against human alpha-fetoprotein (AFP, 1:200, R&D, MAB1368), and mouse monoclonal anti-Albumin (ALB, 1: 200, R&D, MAB1455) overnight at 4°C. On the second day, the cells were washed 3 times with PBS-Tween 20 (0.1%) and incubated with the PE-conjugated as a secondary antibody, goat anti-mouse (1:100; R&D, F0102B) for 45 min at 37 °C in the dark. Subsequently, these matured cells were washed with PBS-%0.1Tween, 3× for 5 min. The nuclei were counterstained with 4', 6-

diamidino-2-phenylindole [(DAPI) (1 µg/ml)] (Sigma, 28718-90-3). All images were taken with a fluorescence microscope (Nikon; Japan).

Imaging with Scanning Electron Microscopy (SEM)

HiPS cell-derived hepatocyte-like cells on the surface of PLLA/PCL polymer were washed three times with PBS and fixed in 2.5% v/v glutaraldehyde for 20 min and dehydrated in a serial of graded ethanol (20% -100%). Subsequently, the cells were dried overnight and sputter-coated with gold before viewing under the SEM (MED010; Baltec). Top images were viewed at an accelerating voltage of 20 Kv using a Hitachi Model-4500 SEM.

Cellular Low-Density Lipoprotein (LDL) uptake assay

Low-Density Lipoprotein (LDL) uptake in matured cells was measured by using the DiI-Ac-LDL staining kit according to the manufacturer's protocol (Biomedical Technologies, BT-904) (Mobarra et al., 2014).

Periodic Acid-Schiff (PAS) stain for glycogen content

Briefly, HLCs-derived cells in the third week were fixed with 4% paraformaldehyde for 20 min at room temperature and then washed with PBS. Fixed cells were oxidized in 1% Periodic Acid (Sigma, 10450-60-9) for 5 min and rinsed 3 times in distilled water. Afterward, induced cells were treated with Schiff's reagent (Sigma, 3952016) for 10-15 minutes. Subsequently, they washed in low pressure running water for 5 minutes and assessed under a light microscope (Olympus, Japan).

Oil red

HLCs-derived cells were fixed with 4% paraformaldehyde for 20 min at room temperature and then washed with PBS and incubated for 45 min with oil red reagent. After that, the cells were washed with running water and analyzed by a light microscope (Olympus, Japan).

Biochemical assays for AFP and Urea production

Conditioned media was collected on days zero, 3, 9 and 18 after the start of differentiation in 2D and 3D cultures. The conditioned media was assayed for ~~A~~alpha-fetoprotein (AFP) production using a chemiluminescence immunoassay kit (Diasorin.UK). Moreover, the urea cell concentration was measured by a Colorimetric Assay Kit (Pars Azmun, 1400029) according to the manufacturer's recommendations. Undifferentiated hiPSCs media were used as controls. The data were normalized based on defined cell number.

Statistical Method

Kolmogorov-Smirnov test was used to check the normality of the data. In the case of normal data, the mean and standard deviation were used to describe the data. To comparison of mRNA expression in iPS and Endoderm on day 7, the independent t-test was used between the two groups of OCT-4 and SOX-17. To compare ALB, AAT, and CK-18, the t-test was used between 2D and 3D groups. As well as, to comparison of AFP in iPS, t-test was used for 2D and 3D groups on day 3, day 9, and day 18. Also, t-test was used to compare urea in iPS between 2D and 3D groups on days 3, 9 and 18. The significance level was taken as $*P < 0.05$.

Results

Scaffold characterization

Hybrid PLLA/PCL scaffold membrane was synthesized by electrospinning and treated by oxygen plasma generator. The morphological characteristics, size and the porous structures of the scaffold were evaluated by SEM micrographs (figure 1A, 1B).

hiPSCs culture and embryonic body formation

The hiPSCs exhibited a tightly packed and flat morphology with sharp edges, large nuclei and limited cytoplasm (Figure 2A). After detaching and transferring in suspension culture medium, the hiPSCs clones formed ovoid structures known as embryonic bodies (EBs) (Figure 2B).

Morphological changes of hiPSCs to HLCs on gelatin-coated plates (2D) and scaffold plates (3D)

Using two-dimensional cultures, the embryonic bodies were seeded into 4-well tissue culture plates for 18 days. As shown in figure 3A, the hiPS cells were attached to the tissue culture plates and began to differentiate into the definitive endoderm by the 3rd day. In the second stage, these endoderm cells changed morphology to the less dense and spindle-like shape that these are the sign of pre-maturity (Figure 3B). However, the combination of recombinant growth factors in final stage resulted in morphological change, with significantly flattened and binucleated cells with large cytoplasm. The features that are a marker for new hepatocytes (Figure 3C). In three dimensional cultures, SEM analysis showed that hiPS cells before differentiation were well attached and penetrated the surfaces of the hybrid membrane (Figure 1C). Viability and the rate of hiPSCs proliferation and differentiation on PLLA/PCL nanofibrous scaffold indicated that matured cells resembled the typical flattened shapes of hepatocytes (Figure 1D). These morphological findings indicate the maturation of hepatic cells.

Endoderm and hepatocyte gene expression

At the beginning and three days after the start of the process, the mRNA of attached cells derived from the EBs were assessed by quantitative PCR. The gene expression of the endoderm marker, FOXA2, was increased significantly after three days, whereas the expression of pluripotent transcription factor OCT-4 was reduced ($P < 0.05$). These results indicate that the hiPSCs could be efficiently differentiated into endoderm in vitro (Figure 4A). As well as, confirming the differentiation status, we found that the gene expression of the hepatocytes specific markers albumin (ALB), alpha-1 antitrypsin (AAT) and cytokeratin-18 (CK18) were increased in 18 days after culturing on the hybrid membrane. A significant up-regulation in the mRNA expression of markers mentioned above was observed in mature cells that had differentiated when grown on PLLA/PCL, compared to those grown in 2D culture which was differentiated on the gelatin-coated plate ($p < 0.05$) (Figure 4B). Overall, these results indicated that synthetic PLLA/PCL scaffold can enhance and improve the efficiency of hiPSCs differentiation to hepatocyte-like cells.

Immunofluorescence and cell staining

Immunostaining analysis was conducted to test whether cell-specific differentiation markers, such as Albumin and alpha-fetoprotein were secreted on 18th days of differentiation and whether this differed by culture conditions. We also examined the ability of the differentiated cells to uptake LDL (Figure 5), and their glycogen content. There was a strong positive staining in HLCs (Figure 6). These results support hepatocyte maturation. As shown in Figure 5, in both 2D and 3D, albumin and AFP, as well as LDL-uptake appeared more rapidly in the number of positive cells for hepatic markers in 3D than 2D cultured cells. Positive staining by Oil Red O and for glycogen granules was detected in the cytoplasm of differentiated HLCs on two-dimensional culture and also those grown on the nanofibrous scaffold (Figure 6).

Alpha-fetoprotein and urea secretion quantity in HLCs

We also investigated the production of other biochemical hepatic markers, alpha-fetoprotein and urea on day 18. The maximum expression of AFP was shown in pre-mature hepatocytes on day 9 before starting the final stage of maturation. A significant difference in production was observed in the pre-matured cells that differentiated on the PLLA/PCL in comparison to the cells in 2D culture (Figure 7A), also urea production continuously increased until day 18 (Figure 7B). This increase in differentiation and then production was more evident in 3D than two-dimensional ($p < 0.05$). Briefly, the control group did not secrete any hepatic markers (data not shown).

Discussion

There has been a significant interest in the use of nanofibers in tissue engineering for stem cells. It appears that the combination of iPSCs and nanofibrous scaffolds have several beneficial effects and potential in the treatment of liver disease (Kazemnejad et al., 2007). A large number of studies have reported that the use of hiPSCs is the best choice for cell transplantation and regenerative medicine in patients with a non-functioning liver (Kehtari et al., 2018; Khademi et al., 2017; Starokozhko et al., 2018). Aloysious and Nair have reported that using 3D culture for the differentiation of various cell types is better than two-dimensional (2D) culture for the differentiation of cells (Aloysious and Nair, 2014), because nanofibrous scaffolds mimic three dimensional structures of the natural microenvironment extracellular matrix (ECM) which enhances effective differentiation (Stendahl et al., 2009). Therefore, nanofibrous scaffolds are increasingly being used for tissue engineering. Preliminary studies have used various scaffolds such as PES (Hashemi et al., 2009; Kazemnejad et al., 2009), PLLA (Feng et al., 2010; Ghaedi et al., 2012) and PCL/collagen/PES (Mahmoodinia Maymand et al., 2017) for hepatocyte differentiation. In the current study, our results demonstrated that hepatic markers are markedly expressed better in hybrid scaffolds than single nanofibrous scaffolds (Mahmoodinia Maymand et al., 2018). L-form of poly-lactic acid (PLLA), is a polyester approved by FDA and L-lactic acid is the product of this scaffolds degradation which is harmless for the body (Islami et al., 2018). Poly (ϵ -caprolactone) (PCL) is a bioresorbable, biocompatible and inexpensive nanofiber, having native tissue-like properties that generally used for tissue regeneration. The hybrid scaffold has several advantages over a single polymer (Shakhssalim et al., 2017). Many studies show that stem cells can differentiate into hepatocyte like-cells by using single nanofibrous scaffolds on gelatin-coated plates or nanofibrous scaffolds, but so far, there have been few studies performed on their effects on the differentiation of hiPSCs to HLCs on the

surface of nano-scaffold hybrids. Therefore, in this study, we used hiPSCs as the source of stem cells and PLLA/PCL hybrid membrane as a new scaffold was used to differentiate into the hepatocyte-like cell. We found that: (1) PLLA/PCL polymer supports the attachment, proliferation, and differentiation of hiPSCs (2) the successful generation of hepatic-like cells from hiPSCs on the hybrid scaffold and (3) the newly derived cells expressed higher levels of liver markers than cells differentiated on gelatin-coated plates and single nanofibrous scaffolds. Our results are consistent with those of Mahmoodinia Maymand et al (Mahmoodinia Maymand et al., 2018) in using SEM, we found that the formed cells aggregated, adhered and penetrated to the scaffold. Our SEM results showed a highly porous and nonwoven architecture that mimics ECM structure, likewise Kinasiewicz *et al* (Kinasiewicz et al., 2008). It was observed that PES membranes support the growth of hepatic cells in 3D culture. This result was also found in our study. Similar to the results of Kazemnejad et al (Kazemnejad et al., 2007) as shown in figure 1, the artificial scaffold formed by the PLLA/PCL hybrid, not only provided a suitable support for hiPSCs proliferation but also allowed the cells to maintain their stability and flexibility during the differentiation process. Our qPCR result showed that hiPSCs expressed the pluripotent stem cell marker OCT-4 and the levels of this protein marker fell after three days of differentiation in endoderm lineage. We also examined the ability of hiPSCs to differentiate into endoderm lineages. As shown in figure 4, endodermic marker FOXA2 successfully was expressed in day 3. This result was consistent with our preliminary reports (Jaafarpour et al., 2018; Jaafarpour et al., 2016) and inconsistent with the study of Mahmoodinia Maymand et al (Mahmoodinia Maymand et al., 2017) that reported the mRNA expression of FOXA2 was 7 fold lower than in our report, this discrepancy may be due to the numbers of days to differentiate into endoderm lineage which in our study was 3 days and study Mahmoodinia Maymand et al (Mahmoodinia Maymand et al., 2017) was 5 days. Moreover, the up-regulation

of albumin, CK18, and AAT specific mRNA further confirm the functionality of hepatocyte-like cells in 3D culture which were significantly more highly expressed than cells induced in two-dimensional cultures. Ghaedi et al (Ghaedi et al., 2012) in a line with our result reported that the expression of albumin and CK-18 were significantly higher in hMSCs cultured on the PLLA nanofibrous scaffold than on cultured in the 2D plate. But the expression level of albumin and CK-18 in cells cultured on PLLA scaffold in her study was significantly lower than our result which cells differentiated on PLLA/PCL membrane, this inconsistency may be due to the type of stem cell and the nanofiber used. Additionally, Mahmoodinia Maymand et al (Mahmoodinia Maymand et al., 2017) have demonstrated that the expression level of marker ALB in hepatocyte differentiated on PES is lower than our result that is 5 folds. These results show that hiPSCs may be better differentiated on hybrid scaffold than single PES nanofibrous membrane. In our study and the report of Kazemnejad et al (Kazemnejad et al., 2007), in the final day of differentiation (Figure 5), Immunohistochemical staining was positive for protein biochemical markers Albumin and AFP in the large fraction of derived hepatocytes in two and three-dimensional cultures. Obviously, the formation of mature hepatocyte-like cells was further confirmed by the positive staining of Oil red and Periodic Acid Schiff in both 2D and 3D (Figure. 6). As well as, secretory markers AFP and urea were investigated as additional markers for hepatocyte-specific function at protein and nitrogen levels in the differentiated cells (Figure 7). The expression of AFP protein in the middle day of differentiation process was dramatically increased, but decreased after 9 days of culture; this reduction in three dimensional culture medium was markedly increased than cells that derived in gelatin plates. This pattern suggests the presence of hepatic progenitors such as oval-shaped cells. As well as, urea production continued to be expressed and increased up to 18 days of culture, especially in cells that cultured and differentiated on PLLA/PCL membrane. This model is agreed with N. Lin1 (Lin et al., 2010) that showed urea production

was increased in bone marrow-derived mesenchymal stem cells into hepatocyte-like cells on the nanofibrous scaffold. Our study has limitations, although we analyzed the potency of hiPS Cell-derived hepatocyte-Like cells on random PLLA/PCL scaffold, the high cost and time do not allow to our team to check the potency of PLLA and PCL differentiation separately and also PLLA/PCL aligned scaffold. Also, further experiments are needed to evaluate the intracellular structures and obtain excellent and desirable liver cells for in vivo regenerative medicine.

Conclusion

We have fabricated a new hybrid PLLA/PCL scaffold membrane and have used this as an approach for the differentiation of hiPSCs to bio artificial hepatocyte-like cells that appears to be highly suitable for this purpose for the first time. This finding suggests that functional matured induced cells on PLLA/PCL scaffold can be used as a successful method for the soft tissue engineering of the liver, tissue engineering therapy, and regenerative medicine.

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Legends

Figure1: Scanning electron microscopy of A, B) unseeded hybrid random PLLA/PCL scaffolds C) hiPSCs-seeded before differentiation on 3D nanofibrous D) Differentiated hiPSCs to HLC on PLLA/PCL membrane. Scale bars a ia 10 μm .and b, c and d are 100 μm .

Figure 2. Phase contrast microscopy images of A) hiPSC colonies on MEFs inactivated feeders B) embryonic bodies (EBs) EBs in hanging culture medium. Scale bars are 100 μm .

Figure 3. Morphological changes of hiPSCs during differentiation on gelatin-coated palates A) began to assemble hiPSCs to endoderm lineage B) pre-matured induced cells C) differentiated hepatocyte-like cells. Scale bars are 100 μm .

Figure 4. Quantitative transcriptional analysis of gene markers. A) pluripotent and endodermal markers at the beginning and after three days of starting the process. B) Relative hepatic gene expression in end-stage derived IPCs. * Significance was defined as $P < 0.05$ and values represent means \pm SD.

Figure 5. Immunocytochemistry staining ALB and AFP protein as well as LDL-uptake the main hepatic markers in end-stage derived cells on gelatin-coated plated (2D) and hybrid scaffold (3D). Nuclei were stained with DAPI. Data are presented as mean \pm SEM. Scale bars are 100 μm .

Figure 6. Staining analysis of matured HLCs include glycogen (PAS) and lipid storage (Oil red) in two (A) and three (B) dimensional culture. Scale bars are 100 μm .

Figure 7. A) Alpha-fetoprotein and **B)** Urea production by hepatocytes at developmental stages. * Significance was defined as $P < 0.05$ and values represent means \pm SD.







